Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity

Maurizio Bocchetta*, Ilaria Di Resta*, Amy Powers*, Raoul Fresco*, Alessandra Tosolini†, Joseph R. Testa†, Harvey I. Pass‡, Paola Rizzo*, and Michele Carbone*§

*Cancer Immunology Program, Department of Pathology, Cardinal Bernardin Cancer Center, Loyola University Chicago, Maywood, IL 60153; †Human Genetics Program, Fox Chase Cancer Center, Philadelphia, PA 19111; and ‡Aerodigestive Program, Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201

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Mesothelioma, a malignancy associated with asbestos, has been recently linked to simian virus 40 (SV40). We found that infection of human mesothelial cells by SV40 is very different from the semipermissive infection thought to be characteristic of human cells. Mesothelial cells are uniformly infected but not lysed by SV40, a mechanism related to p53, and undergo cell transformation at an extremely high rate. Exposure of mesothelial cells to asbestos complemented SV40 mutants in transformation. Our data provide a mechanistic explanation for the ability of SV40 to transform mesothelial cells preferentially and indicate that asbestos and SV40 may be cocarcinogens.

Malignant mesothelioma (MM) is a tumor of the serosal lining the pleural, pericardial, and peritoneal cavities that causes about 2,500 deaths per year in the United States (1). MM arises from the malignant transformation of mesothelial cells, which are undifferentiated cells representing the adult remnants of the surface coelomic mesoderm (1). Although MM has been associated with past exposure to asbestos fibers, the mechanisms through which asbestos causes mesothelial cell transformation are unclear. The capacity of asbestos to induce autophosphorylation of the epidermal growth factor receptor, which leads to activation protein-1 activity in human mesothelial cells (HM; ref. 2); the production of reactive oxygen species by cells exposed to asbestos (3); and the local and systemic immunosuppressive effects of asbestos (4) may all contribute to carcinogenesis (1). Other factors act alone or synergistically with asbestos in causing MM, because only 5–10% of individuals exposed to high levels of asbestos develop MM, and 10–20% of MM occurs in individuals with no known exposure (1).

Recently, simian virus 40 (SV40) has been associated with human mesothelioma and brain and bone tumors (reviewed in refs. 1 and 5–7). SV40 (5–8) is a DNA tumor virus encoding two transforming proteins (the large tumor antigen, or Tag; and the small tumor antigen, or tag), and three capsid proteins (VP1–3). Tag is the replicase of SV40. Expression of Tag in the absence of cell lysis leads to cellular transformation through several mechanisms, including Tag-mediated inhibition of cellular p53 and Rb family proteins, induction of insulin-like growth factor-I and its receptor, and the direct mutagenic effect of Tag. SV40 tag enhances Tag functions by inhibiting protein phosphatase 2A, contributing to malignancy (1, 9).

SV40 infects cells from different species, and the cell type determines the outcome of SV40 infection (5–8). Permissive monkey cells support SV40 replication, which results in cell lysis. In nonpermissive rodent cells SV40 DNA cannot be replicated, and cells are not lysed and can be transformed. Human cells are termed semipermissive because only a fraction of cells express SV40 Tag after infection, these infected cells are lysed, and cell transformation is a very rare event.

SV40 is highly oncogenic in rodents (5–7). We found that intracardial injection of SV40 induced MM in 60% of hamsters, whereas intrapleural injection caused 100% incidence of MM in 3–7 months (10). In contrast, asbestos caused MM in a minority of intrapleurally injected hamsters or other animals after a long latency (1, 11). About 60% of human MM contains SV40 DNA (1, 5–7). Tag immunostaining demonstrated viral expression in the MM cells and not in nearby stromal cells (5–7). This specificity was confirmed by recent microdissection experiments that detected SV40 in MM cells, but not in nearby stromal cells microdissected from the same slides (12). In MM and in brain tumors, Tag binds and inhibits p53 and pRb, possibly contributing to carcinogenesis (13–15). This possibility was supported by recent experiments demonstrating that Tag expression was required for the maintenance of the transformed phenotype of cell lines derived from SV40-positive MM, an effect related to the inhibitory binding of Tag with p53 (16). These results, and the fact that SV40 can be found in nonneoplastic mesothelium (12), suggest that HM might be unusually susceptible to SV40 infection and transformation. Why HM would be targeted by SV40, how infection of semipermissive human cells could lead to malignant transformation, and the possible interaction with asbestos have remained unknown.

Materials and Methods

Cells. We used three different primary mesothelial cell cultures. Two (HM1 and HM2) were from two separate patients who accumulated pleural fluid because of congestive heart failure; a third culture (HM3) was established by pooling cells from five patients with congestive heart failure or liver disease. HM were used at passages 3–7; HM became senescent at passage 8–9. The identity of HM was established morphologically and confirmed by electron microscopy (EM) (presence of long microvilli and perinuclear tonofilaments) and by positive immunostaining for cytokeratin, HBME-1, and calretinin, and negative staining for LeuM1, BerEp4, B72.3, and carcinoembryonic antigen. After 2 weeks in culture, contaminating cells died, and 100% of cells stained positive for calretinin and were expanded and then used for the experiments described in the text. In parallel, we used three different cultures of primary human diploid fibroblasts (HF) as a control: WI38 and MRC-5, both fetal lung HF, which

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Abbreviations: MM, malignant mesothelioma; HM, human mesothelial cells; SV40, simian virus 40; Tag, SV40 large tumor antigen; tag, SV40 small tumor antigen; EM, electron microscopy; HF, human fibroblasts.

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§To whom reprint requests should be addressed at: Loyola Medical Center, Cardinal Bernardin Cancer Center, Room 205, 2160 S. First Avenue, Maywood, IL 60153. E-mail: mcarbon@orion.it.luc.edu.

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were used in our experiments at passages 17–18, and CCD1069Sk breast HF from a 70-year-old woman, which were used at passages 6–7, all from the American Type Culture Collection. WI38 were chosen because they were used extensively in the past to study SV40 infection of human cells and because of their lung origin; MRC-5, because of their lung origin; and CCD1069Sk, because of the early passage available and to test fibroblasts from an adult. Primary HM were established and subsequently grown in tissue culture in DMEM containing 20% FBS. All fibroblast cell cultures were grown in DMEM containing 10% FBS.

Infection Experiments. SV40 infection of the three different primary HM cultures was carried out at intervals of 2–3 months in six dish wells, using nonarchetypal SV40 strain 776, which contains two 72-bp repeats in the enhancer region. Strain 776 is the SV40 type most commonly detected in MM (6).

Immunostainings were repeated two or more times for each type of HM or HF, as described (13). Furthermore, cells were routinely scored for Tag expression by immunostaining in each infection experiment. The results were reproducible. To determine SV40 DNA and protein expression in the infected cultures, the medium and cell layers were assayed separately.

Medium. Forty-eight hours after infection, the culture medium was removed and centrifuged at $10,000 \times g$ for 5 min to remove cell contaminants. The supernatants were then centrifuged at $100,000 \times g$ for 5 h to collect viral particles, and the pellets were suspended in water.

Cells. Cells were trypsinized, washed, counted, and snap-frozen. DNA was extracted from both cell and ultracentrifugation pellets according to the standard Hirt extraction procedure. DNA suspensions (volumes were normalized for number of Tag-positive cells) were loaded onto 1% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled SV40 probe as described (17). To determine the level of VP1 in the ultracentrifugation pellets, the samples were suspended in 100 μl of 1× SDS buffer [50 mM Tris·HCl (pH 6.8)/100 mM DTT/2% SDS/0.1% bromophenol blue/10% glycerol], incubated at 100°C for 5 min, and loaded onto an SDS/10% polyacrylamide gel (after normalization for number of Tag-positive cells). VP1 levels in the cells were assayed from cell extracts in lysis buffer [150 mM NaCl/1% Nonidet P-40/2 mM EDTA/1 mM DTT/50 mM Tris·HCl (pH 8.0)/10 μg/ml each of chymostatin, leupeptin, aprotinin, and pepstatin/1 mM PMSF]. Lysates were incubated on ice for 15 min, sonicated at 4°C, and cleared by centrifugation. Protein concentrations were measured by using the Bradford assay (Bio-Rad). SDS buffer (1× final concentration) was added to 100 μg of cell extracts, and samples were incubated at 100°C for 5 min and loaded onto an SDS/10% polyacrylamide gel. AB-597 mouse monoclonal antibody for VP1 was a gift from F. J. O'Neil (Department of Veterans Affairs Medical Center, University of Utah, Salt Lake City).

p53 Studies. The p53 of HM was down-regulated by cultivating cells in medium containing 5 μM all-phosphorothioated oligonucleotides (IDT, Coralville, IA). The sequence of the antisense oligo specific for the *p53* mRNA was 5′-GCGGCTCCTCCATGGCAGTGACC-3′. This oligo is complementary to the *p53* start codon and to 10 nucleotides upstream and downstream from it. Control experiments were performed by using a 23-mer oligo with randomized positions, the G + C content of which was 69% (equal to that of the anti-p53 oligo). Down-regulation experiments were performed in T25 flasks with 2.5 ml of medium supplemented with different oligos. To investigate the association of SV40 Tag with p53 in HM treated with oligos, cells were treated with 5 μM either scrambled or antisense *p53* oligos for 48 h. At this time cells were infected with SV40 (multiplicity of infection of 10), providing at the same time a fresh aliquot of oligos to each sample. Cells were lysed 72 h after infection, and 100 μg of total cell lysates was tested for p53 expression by Western blotting. The remaining cell lysates (800 μg of total cell proteins) were precleared with protein A/Trisacryl (Pierce); incubated with monoclonal anti-Tag pAb-419 (AB-1) (Oncogene Science), for 1 h at room temperature, precipitated overnight at 4°C with protein A/Trisacryl, run on an SDS/10% polyacrylamide gel, and blotted onto nitrocellulose membrane. The latter was probed with monoclonal anti-Tag pAB-416 (AB-2) (Oncogene Science), followed by incubation with a horseradish peroxidase-conjugated monoclonal anti-mouse IgG (Oncogene Science). To visualize p53, the membrane was stripped 1 h at 70°C in 100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris·HCl (pH 6.7) and probed with DO-1 anti-p53 directly conjugated with horseradish peroxidase (Santa Cruz Biotechnology) (this procedure was necessary to avoid cross-hybridization between the secondary antibody and the heavy chain of the mouse IgG used for immunoprecipitation, which runs at 54–55 kDa). The reactions were visualized by enhanced chemiluminescence.

Transformation Experiments: Infections. Focus assays were performed in six dish wells. Infected and noninfected (controls) HM (HM1–3) and HF were followed for 12 weeks, when cells that were not transformed sloughed off. Sixteen independent foci of HM were taken, and all of them were successfully established in tissue culture. Six independent foci were taken from CCD1069Sk, but these foci did not grow. Control untreated HM could not be passed more than eight times in tissue culture.

Transformation Experiments: Transfections. Transfections required a large number of cells that were available only from HM3, which was a pool of five different primary HM. WI38 were used as control. Each transfection was performed in six parallel dishes; the cells were evaluated for 12 weeks for focus formation. Crocidolite asbestos (U.S. National Institute of Environmental Health Sciences) was diluted and prepared as described (3). The susceptibility of HM and HF to asbestos was tested at various concentrations of crocidolite, 0.1, 0.5, 1.0, 2.5, and 5.0 μg/cm^2, in three separate experiments (two in HM3; one with HM2); WI38 HF were used as a control. Transformed foci did not develop over a 2-month observation period, at which time all HM were dead. Plasmids pEGFP-N1 (CLONTECH) and plasmids pw2, pw2dl, pw2t, and p101 (from K. Rundell, Department of Immunology and Microbiology, Northwestern University, Chicago) (18) were used in transfections performed with the LipofectAmine Plus kit (GIBCO/BRL). Asbestos fibers were dispersed in the tissue culture medium to achieve final concentrations ranging from 0.1 to 5 μg/cm^2. The final count of the number of foci was taken 8 weeks after transfection.

Results

Infection Experiments. To test the susceptibility of HM to SV40, we infected at a multiplicity of infection of 10, primary HM (three different cultures, HM1–3) and primary HF (control) (three cultures, WI38, MRC5, and CCD1069Sk) with SV40 (Fig. 1). Only a fraction of HF (about 20% of WI38 and 0.5–1% of MRC5 and CCD1069Sk) expressed Tag 48 h after infection. The percentage of Tag-positive HF increased to about 20% 7 days after infection, and there were no appreciable differences among different HF at that time and thereafter. Instead, the majority (about 95%) of HM expressed Tag as early as 24 h after infection and thereafter. A cytolytic effect was evident by light microscopy in HF 48–72 h after infection (Fig. 1), and it was confirmed by EM (Fig. 2*A*). In contrast, whereas HM contained complete viral particles (Fig. 2*B*), cell lysis was rare. Seven days after infection, about 20% of HM were lysed, while most of the other cells expressed Tag with no detectable morphological changes. Instead, at the same time almost all Tag-positive HF were lysed. Twelve weeks after infection, 1–5% of surviving HF expressed Tag and subsequently lysed (Fig. 1). At this same time, HM were

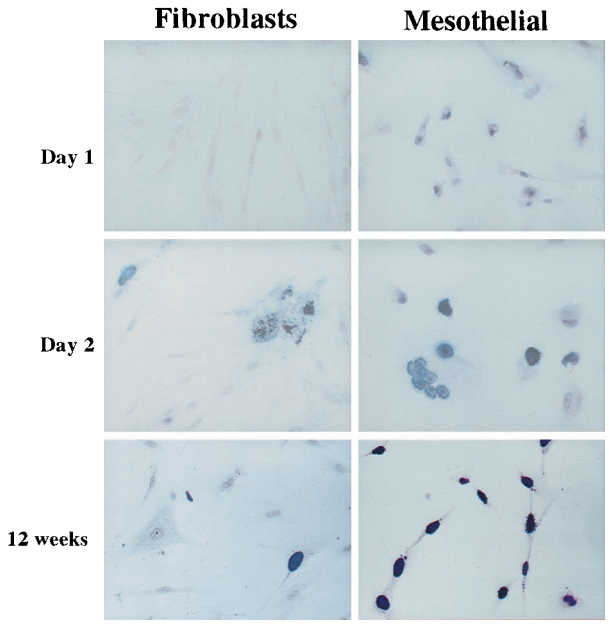

Fig. 1. Tag immunostaining. WI38 HF (*Left*) and HM3 (*Right*), at the indicated time after infection. Similar results were obtained with the other cells. No substantial differences were observed among HM1–3. In HM, the nuclear staining was punctate at 24 h, then it became granular, with the formation of intranuclear bodies, and finally obscured the entire nucleus. In HF, only a fraction of cells expressed Tag. These cells formed ill-looking cell clumps and giant cells, with clear evidence of cytopathic effects, such as vacuolization and lysis. (Original magnification, ×400.)

healthy and uniformly expressed Tag, and only rare cells were lysed (Fig. 1). Thus HF showed the expected "semipermissive" type of infection characteristic of human cells (5–8), including some differences among primary cultures. HM, instead, were uniformly infected, and no differences in their susceptibility to SV40 were observed among three primary HM cultures (HM1–3). In HM, Tag was detectable earlier than in HF, viral particles

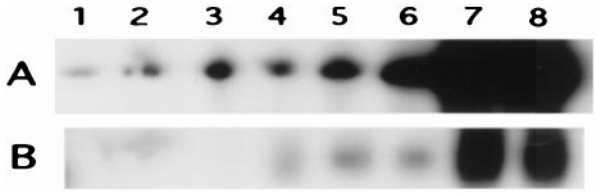

Fig. 3. Southern blot for SV40 DNA in HM3 and WI38. (*A*) SV40 DNA extracted from the cells. (*B*) SV40 DNA recovered in the tissue culture medium. Lanes: 1 and 2, HM 48 h after infection; 3 and 4, HF 48 h after infection; 5 and 6, HM 72 h after infection; 7 and 8, HF 72 h after infection. Each lane represents an independent infection experiment. Almost identical results were obtained with HM2 and MRC-5 HF. HM1 and CCD1069Sk were not tested. The different amounts of DNA (see text) were determined by Cherenkov counting of the individual lanes. DNAs were normalized for number of Tag-positive cells.

were seen by EM, but cell lysis was not prominent. EM suggested that fewer viral particles were produced in HM compared with HF. EM, however, is not a quantitative test. To verify if less virus was produced in HM, we measured the amount of SV40 DNA and of the major capsid protein VP-1 in infected HF and HM.

Southern blot hybridization of Hirt extracts from HF and HM (Fig. 3*A*) and from the corresponding tissue culture medium (Fig. 3*B*) with an SV40-specific probe 48 and 72 h after infection revealed that at 72 h HF contained 10 times more SV40 DNA than did HM (Fig. 3*A*, lanes 5–8). Seventy-two hours after infection, the culture medium from HF contained 12 times more SV40 DNA compared with HM, probably the result of viral release after cell lysis (Fig. 3*B*, lanes 5–8). Western blot analyses for VP1 revealed large amounts of VP1 at both 48 and 72 h after HF infection, confirming active viral replication. At the same time points HM contained barely detectable amounts of VP1 (Fig. 4*A*). Similar results were obtained by testing the tissue culture medium of these cells, supporting cell lysis in HF (Fig. 4*B*). These observations indicated that HM were able to support SV40 replication, but at a lower level compared with HF, and that cell lysis was rare in infected HM.

p53 Studies. Next we studied the mechanisms that might account for the unusual pattern of SV40 infection in HM. Because p53 down-

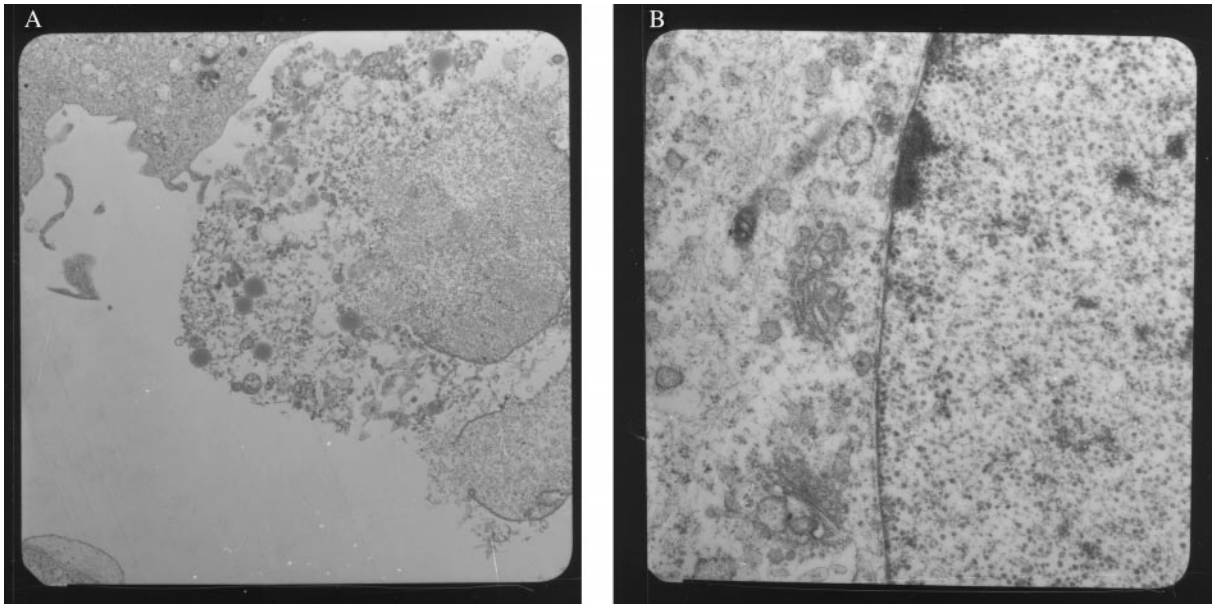

Fig. 2. EM of WI38 HF (*A*) and HM3 (*B*) infected with SV40 72 h earlier. Note that infected HF are lysed and full of viral particles (the individual viral particles are not clearly visible at this magnification). HM, instead, have intact nuclear membrane, and viral particles (round structures) are seen only in the nucleus (right side of the photograph). (Original magnifications: *A*, ×4, 400; *B*, ×20,000.) The same results were obtained when HM2 were used; HM1 were not tested.

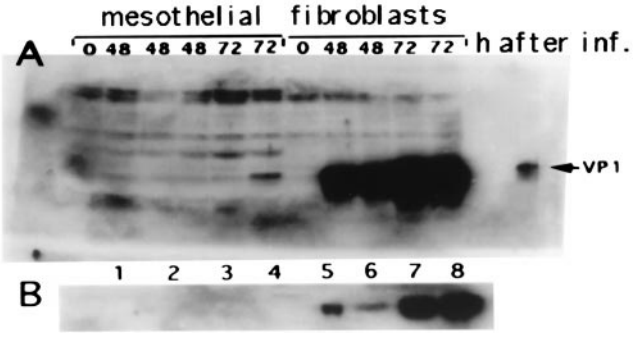

Fig. 4. Western blot for SV40 VP1 in HM3 and WI38 after infection with SV40. Blots were developed by enhanced chemiluminescence. (*A*) VP-1 in the total cell extracts (100 μg per lane). (*B*) VP1 in the tissue culture medium (samples were normalized for number of Tag-positive cells). Lanes: 1 and 2, HM 48 h after infection; 3 and 4, HM 72 h after infection; 5 and 6, HF 48 h after infection; 7 and 8, HF 72 h after infection. Each lane represents an independent infection experiment. Almost identical results were obtained with all of the other HM and HF. Cell extracts were normalized for number of Tag-positive cells.

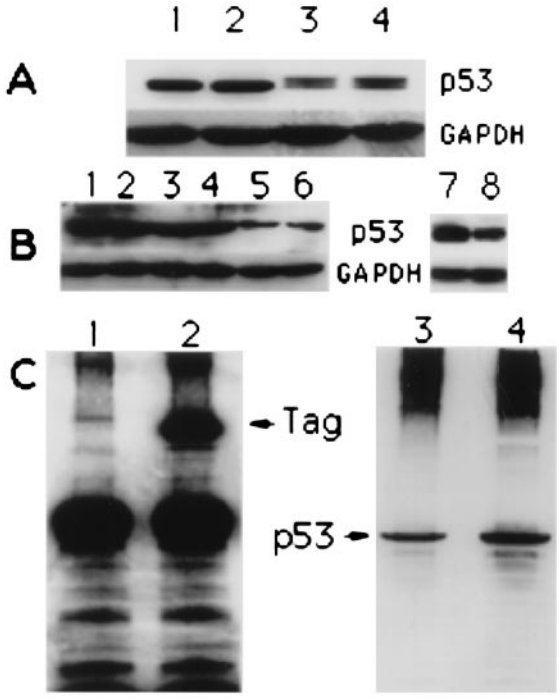

Fig. 5. (*A*) p53 expression in HM and HF. One hundred micrograms of total protein extracts was loaded into each lane. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Lanes: 1, HM2; 2, HM3; 3, WI38; 4, CCD1069Sk [HM1 and MRC5 (not shown) produced almost identical results]. (*B*) Lanes 1–6, p53 expression in untreated HM (lanes 1 and 2), HM treated with 5 μM scrambled oligo (lanes 3 and 4), and HM treated with 5 μM antisense *p53* oligo (lanes 5 and 6). Cells were harvested and lysed 48 h after the onset of treatment; 100 μg of total protein extracts was loaded per lane (each lane represent an independent experiment). Lanes 7 and 8, p53 expression in HM 5 days after treatment (and 72 h after SV40 infection) with scrambled oligos (lane 7) and antisense p53 (lane 8). (*C*) Lanes 1 and 2, Tag immunoprecipitation in SV40-infected HM. Lane 1, scrambled control oligo-treated HM; lane 2, antisense p53-treated HM. Tag was precipitated with the monoclonal anti-Tag AB-1 (Tag amino terminus). The membrane was probed with the monoclonal anti-Tag AB-2 (Tag carboxyl terminus), followed by monoclonal anti-mouse IgG conjugated with horseradish peroxidase. Lanes 3 and 4, Tag/p53 coimmunoprecipitation. The membrane shown on the left was stripped of antibodies and probed with the monoclonal anti-p53 DO-1 directly conjugated with horseradish peroxidase. Lane 3, control oligo-treated HM; lane 4, antisense p53-treated HM.

regulates Tag replicase activity (19, 20), and Tag binds to p53 in MM (13), we investigated the possibility that this association contributed to the limited replication of SV40 in HM. HF (all three cultures) contained two forms of p53 distinguishable by SDS/PAGE (Fig. 5*A*), probably the result of different conformational or phosphorylation states (21, 22). HM (all three cultures) contained only a single p53 band. We sequenced all 11 exons of *p53* (as described in ref. 13) in HF WI38, HM1, and HM2. All contained wild-type *p53* (not shown). Western blot analysis before infection (Fig. 5*A*) showed that HM expressed approximately 4 times more p53 than did HF. We hypothesized that the higher expression of p53 in HM inhibited SV40 replication. To test this mechanism, HM were treated with an antisense oligo for *p53* before infection. A control oligo containing a random sequence of nucleotides, but with the same GC content as the antisense oligo, was used in parallel experiments. Western blot analyses showed that antisense-treated HM expressed about 1/5 as much p53 as did the control 48 h after treatment (Fig. 5*B*, lanes 1–6). Infection of HM was carried out 48 h after treatment with the antisense or the control oligonucleotide. Seventy-two hours after infection, and 5 days after oligo treatment, antisense-treated HM expressed 1/3 as much p53 as did controls (Fig. 5*B*, lanes 7–8). At this time, immunoprecipitation of Tag showed a marked increase in the amount of Tag in antisense-treated cells compared with controls (Fig. 5*C*, lanes 1–2). Coimmunoprecipitation of Tag and p53 was also observed (Fig. 5*C*, lanes 3–4). Antisense-treated cells synthesized (on average) about 4 times more SV40 DNA than the HM cells exposed to the control oligo (Fig. 6*A*). Furthermore, cell lysis was specifically observed in about 50% of HM treated with antisense *p53* (Fig. 6*C*) and was not observed in HM transfected with scrambled control oligo (Fig. 6*B*). Consistently higher levels of VP1 were detected in the medium of cells treated with antisense *p53* compared with controls (not shown). These results indicate that p53 plays an important role in down-regulating the replication of SV40 genomes in infected HM and in preventing HM lysis.

Transformation Experiments. Prolonged expression of Tag in HM in the absence of cell lysis may have caused long-term biological effects. To test this possibility, we infected HM and HF and monitored the cells for 12 weeks for focus formation. Six weeks after infection, transformed foci arose from infected HM (Table 1). No foci developed from a total of 10^7 infected WI38 HF or from a total of 10^7 infected MRC-5 HF, whereas six foci developed from 7.4×10^7 infected CCD1069Sk HF. Cells from these six HF foci could not be established in tissue culture. Instead, the average transformation frequency (number of foci per infected HM) was very high (2×10^{-4}), and no substantial differences were observed among different primary HM. About 8 weeks after infection the foci were visually detectable (1-mm diameter), and 16 independent HM foci were put in culture; all grew. Two of these foci grew for 30 passages and then entered a crisis leading to cell death. The other 14 HM foci were immortal, did not go through crisis, and have been passed up to 76 times in tissue culture. Clonality was confirmed by comparative genomic hybridization analyses of DNA from several transformed HM foci that demonstrated distinct chromosomal alterations (not shown). Individual foci/clones demonstrated various degrees of serum independence (growth in medium containing 1–5% FBS) and plating efficiencies (1–20%) in soft agar (uninfected HM did not form colonies on soft agar and did not grow in low serum). Southern blot experiments showed episomal SV40 DNA in these foci (not shown). Transformed HM released infectious viral particles throughout their growth in tissue culture, because medium from HM cultures (tested at tissue culture passages 4 and 55) induced Tag staining, vacuoles, and lysis of CV-1 monkey kidney cells. Control medium from noninfected HM did not cause any change in CV-1.

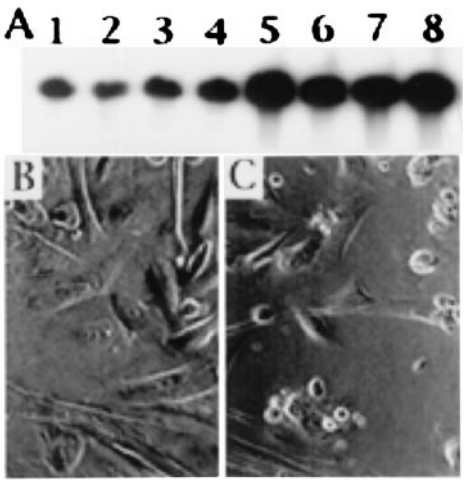

Fig. 6. (*A*) Southern blot analysis of SV40 DNA synthesis in SV40-infected HM3 cells treated with a control scrambled oligo (5 μM) (lanes 1–4) and treated with antisense *p53* (5 μM) (lanes 5–8). Almost identical results were obtained with HM2; HM1 were not tested. (*B* and *C*) HM2 treated with control scrambled oligo (*B*; 25 μM); or antisense *p53* (*C*; 25 μM) and infected 48 h later with SV40. Cells are shown 72 h after infection. In *C*, note empty spaces because of lysis in cells treated with antisense *p53*. Little or no cell lysis was observed in cells treated with control oligos (*B*). (Original magnification, ×400.)

Transfections with SV40 ori-Minus Plasmids with or Without Asbestos. To verify that differences in the rate of SV40 replication in HM and HF played an important role in the different rates of transformation observed (Table 1), we transfected HM and HF with the SV40 origin of replication-defective (ori−) plasmids harboring various combinations of the SV40 early genes (Table 2). Both HF and HM developed foci when transfected with ori− plasmids containing SV40 Tag and tag. Foci did not develop in cells transfected with the other plasmids, which expressed only one of the two SV40 tumor antigens, or in those transfected with the control plasmid (Table 2).

Because human cancer is multifactorial, in parallel, we performed similar transfections in the presence of crocidolite asbestos (the type of asbestos most closely associated with MM) to test for cocarcinogenesis. In controls, HM and HF were exposed to concentrations of crocidolite ranging from 0.1 to 5.0 $\mu g/cm^2$. Asbestos was tolerated well by HF in a 60-day period of exposure, and cell death was rare. Instead, crocidolite induced cell death in HM at all concentrations tested. When we used 2.5 $\mu g/cm^2$ of crocidolite, the minimal concentration shown to induce activation protein-1 in HM (3), about 50% of HM died within 7 days of exposure. No foci developed from HM or HF exposed to crocidolite alone at any concentration in all experiments performed (Table 2). When HF were transfected with both Tag and tag and were exposed to crocidolite 3 days later (2.5 $\mu g/cm^2$), a higher number ($P < 0.05$) of foci developed compared with HF transfected in the absence of crocidolite (Table 2). Although crocidolite was toxic for HM, the surviving cells transfected with Tag and tag appeared to be more prone to cell transformation, but this effect was difficult to quantitate because of asbestos-induced HM death (Table 2). It is noteworthy that foci developed in HM transfected with pw2dl (Tag+ tag−) and exposed to crocidolite (Table 2), because neither crocidolite alone nor the transformation-defective SV40 plasmids alone could transform human cells in these (Table 2) or in previous experiments (18).

Table 1. Focus assay after infection of HM with SV40 in separate experimentsCell type

Cell type	No. of foci per well
HM1	9.83 ± 1.835
	10.17 ± 3.125
HM2	10.50 ± 5.577
	12.33 ± 4.599
	10.00 ± 4.147
HM3	10.67 ± 2.732
	8.67 ± 2.340

Cells (3×10^5) in six separate wells were infected in each experiment. No foci developed in parallel infections from a total of 10^7 WI38 or 10^7 MRC-5 in three different experiments. Six foci developed from a total of 7.4×10^7 CCD1069Sk.

Discussion

We found that HM are uniquely susceptible to SV40. SV40 infection of HM did not follow the so-called semipermissive infection thought to be characteristic of human cells. Rather, HM were uniformly infected, Tag was expressed in 100% of the cells, but HM produced only 1/10 as much SV40 DNA and VP1 as HF did (Figs. 1–4). We found that the higher levels of p53 present in HM compared with HF bind Tag and inhibit Tag-induced SV40 replication, limiting cell lysis. This possibility is supported by the observation that antisense *p53* led to increased levels of Tag, increased SV40 replication, and subsequent HM lysis (Figs. 5 and 6). Prolonged expression of Tag in 100% of the HM exposed to SV40 led to a rate of transformation more than 1,000 times higher than that in HF (Table 1).

The transfection experiments confirmed that SV40 replication and subsequent cell lysis were the limiting factors in focus formation in infected HF, because foci developed in the same HF transfected with ori− Tag+ tag+ plasmids (Table 2). However, additional factors influence the susceptibility of HM to SV40 transformation, because the number of foci was significantly higher in HM compared with HF ($P < 0.01$; Table 2).

The HM foci derived from infections showed a transformed phenotype, because they grew in low serum and in soft agar, and 16 of 16 could be established in tissue culture. In contrast, the 6 foci that developed from infected HF could not be established in tissue culture. Fourteen of 16 HM foci were immortal and have been passed up to 76 times in tissue culture without going through "crisis." Crisis is a period in which the cells become growth arrested after an initial extension of their lifespan caused by a carcinogen, such as SV40. Occasionally, a subclone of cells develops additional genetic alterations, and the cells escape crisis and become immortal. Human cells exposed to SV40 may become immortal without going through crisis, but this is thought to be a rare event [less than 5% of foci (8)]. The high rate of immortal HM clones we obtained that did not undergo "crisis" underscores the unusual susceptibility of HM to SV40.

The presence of episomal SV40 in the HM foci may appear unusual because in SV40-transformed human cells, SV40 is frequently integrated (5–8). There are, however, occasional reports of episomal SV40 in some transformed human cells (23–25), and human mesotheliomas and brain tumors contain episomal SV40 (5, 6). Our HM clones contained episomal SV40 (not shown) and continued to release low amounts of infectious virus in the tissue culture medium even after 55 passages, indicating low levels of SV40 replication. It is possible that most HF cannot survive with episomal SV40 because in these cells SV40 replicates actively and lyses the cells. Therefore, only the rare HF in which SV40 becomes integrated can be transformed. There is no such selective pressure for HM, inasmuch as these cells can prevent cell lysis by limiting SV40 replication because of high levels of p53.

The overall effect of crocidolite asbestos on cell transformation may appear modest (Table 2) compared with SV40. The role of asbestos in causing MM has been firmly established epide-

Table 2. Focus assays after transfection with plasmids containing various SV40 constructs with or without asbestos

Cell line	Treatment	Frequency of focus formation*
Mesothelial cells	Asbestos fibers	—†
Fibroblasts	Asbestos fibers	—
Mesothelial cells	Transfection T+t+	$4.6 \times 10^{-4} \pm 0.53 \times 10^{-4}$
Fibroblasts	Transfection T+t+	$6 \times 10^{-6} \pm 2.06 \times 10^{-6}$
Mesothelial cells	Transfection T+t−	—
Fibroblasts	Transfection T+t−	—
Mesothelial cells	Transfection T+t+ and asbestos	$6 \times 10^{-4} \pm 3.46 \times 10^{-4}$
Fibroblasts	Transfection T+t+ and asbestos	$9.3 \times 10^{-6} \pm 0.66 \times 10^{-6}$
Mesothelial cells	Transfection T+t− and asbestos	$4 \times 10^{-4} \pm 2.31 \times 10^{-4}$
Fibroblasts	Transfection T+t− and asbestos	$3.6 \times 10^{-6} \pm 0.88 \times 10^{-6}$

*Number of transformed foci per treated cell.

†No foci developed from 10^7 cells exposed to asbestos. In addition, no foci developed from 10^7 cells transfected with the plasmids T−t+, or T−t− with or without asbestos, or from control cells. The following plasmids were used: pw2, expressing both Tag and tag (T+t+); pw2dl, which expresses Tag alone (T+t−); pw2t, which expresses tag alone (T−t+); and pw101, the negative control, expressing only a mutated tag that is biologically inactive because it does not bind protein phosphatase 2A (18) (T−t−).

miologically; however, it has been difficult to reconcile the epidemiological findings with the inability of asbestos to transform mesothelial cells in tissue culture (1) (Table 2). *In vivo*, the immunosuppressive activity of asbestos (4) may be important in tumor development and in preventing immune lysis of Tag-positive HM. Furthermore, asbestos induces the production of oxygen radicals by macrophages (2), which may promote gene alterations and carcinogenesis. Thus it is possible that *in vivo*, asbestos has stronger effects on carcinogenesis than *in vitro*. We found that *in vitro*, asbestos and SV40 are cocarcinogens, because more foci developed in HF transfected with SV40 in the presence of asbestos, and because foci developed from cells transfected with SV40 tag mutants in the presence of asbestos. In parallel experiments without asbestos, these same mutants did not cause transformed foci (Table 2), confirming the requirement for both Tag and tag for transformation of human cells (18). Mesotheliomas containing SV40 sequences with deletions in the tag coding sequences have been detected (17), but their significance was unclear. Our new findings suggest that in the presence of asbestos, SV40 tag mutants may contribute to carcinogenesis. Induction of activation protein-1 by crocidolite and SV40 tag has been linked to mesothelioma pathogenesis (2, 3) and SV40 infection (1), respectively. This mechanism may account for the ability of crocidolite and SV40 tag mutants to complement each other in cell transformation.

In conclusion, our findings address two important questions about the association of SV40 with MM. First, our data provide a mechanistic explanation for the ability of SV40 to transform HM preferentially. The enhanced susceptibility of HM to SV40 infection and the ability of HM to resist SV40-induced cell lysis, a mechanism related to p53 levels, permits prolonged Tag expression in a large number of cells, which leads to the development of an increased number of transformed foci (2×10^{-4}) in SV40-infected HM compared with none or only 0.8×10^{-7} in infected HF. Second, we provide a model indicating that asbestos and SV40 may be cocarcinogens. These data are critical conceptually, as SV40 is found frequently in MM patients with a history of asbestos exposure (26). Moreover, SV40 is specifically detected in MM cells and not in nearby stromal cells or lung cancer biopsies (12), and the presence of SV40 contributes to the maintenance of the transformed phenotype (16) and may negatively influence prognosis (27). MM may be a new example of cocarcinogenesis between a virus (SV40) and a ubiquitous environmental carcinogen (asbestos).

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